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GENUS *SALVIA* – ECOSYSTEM FOR ISOLATION OF LACTIC ACID BACTERIA

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ABSTRACT

In recent years there has been a trend of increased interest in lactic acid bacteria (LAB) isolated from non-dairy sources due to their diverse metabolic profile and unique flavor-forming activities. This study presents the possibility of using microbial diversity of individual plant parts (flower, leaf and steam) of each species of the genus *Salvia* for isolation of LAB with new metabolic activities, suitable for their potential inclusion in starter cultures. After screening 800 microbial isolates derived from five species of the genus *Salvia* and subsequent multiple transfer and growth in selective media, 460 single bacterial colonies were isolated. The data from the required and confirmatory tests established that 56 single colonies showed phenotypic identity (Gram-positive, catalase-negative, oxidase-negative and indole-negative) with the group of LAB. All were classified as homofermentative cocci. 82.2% of the plant-derived lactobacteria synthesized L(+)-lactic acid, but a minor part (11.8%) synthesized both isomeric forms of lactic acid. Almost all isolates have a wide pH and temperature range as well as high halotolerance. Using genotype-based methods such as 16S rDNA sequencing, the plant-derived bacterial isolates were identified as: *Enterococcus faecium*, *Enterococcus casseliflavus*, *Enterococcus mundtii*, *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus*.

Keywords: Lactic acid bacteria, Genus *Salvia*, Isolation, 16S rDNA

INTRODUCTION

Lactic acid bacteria (LAB) include a wide variety of cell types and physiological and biochemical characteristics. They are predominantly represented as a non-taxonomic heterogeneous group of Gram-positive and non-spore forming facultative anaerobic bacteria. LAB could be isolated from traditional sources - raw milk, dairy products and fermented foods (**Kimoto *et al.*, 2004; Tamang *et al.*, 2005; Nomura *et al.*, 2006; Kostinek *et al.*, 2007; Tanasupawat *et al.*, 2007; Venturi *et al.*, 2012; Abegaz, 2014; Gad *et al.*, 2014; Guley *et al.*, 2014/15)** or from alternative sources - fecal samples, soils and plants **(Hartnett *et al.*, 2002; Magnusson *et al.*, 2003; Cock and De Stouvenel, 2006; Siezen *et al.*, 2008; Trias *et al.*, 2008; Di Cagno *et al.*, 2009 ; Cakir, 2010; Chen *et al.*, 2010; Venugopalan *et al.*, 2010; Baradaran *et al.*, 2012; Emerenini *et al.*, 2013; Fhoula *et al.*, 2013; Nguyen *et al.*, 2013; Alemayehu *et al.*, 2014).** In recent years there has been a trend of increased interest in LAB isolated from non-dairy sources (plant origin) due to their diverse metabolic profile and unique flavor-forming activities. Plant-derived strains of lactobacteria have demonstrated tolerance to high pH values and salt concentrations, ability to ferment various types of carbohydrates and a high level of stress resistance compared to those of dairy origin. Furthermore, no significant differences were noted in the fermentation characteristics and profiles of enzymes, such as lipases, peptidases and phosphatases, required for obtaining various fermented dairy products with plant-derived and commercial strains of lactobacteria **(Nomura *et al.*, 2006; Michaylova *et al.*, 2007; Siezen *et al.*, 2008; Venugopalan *et al.*, 2010)**.
The search for new solutions to improve health fermented food starters and to expand the opportunities for comprehensive use of the biological potential of LAB has provoked the idea to exploit the unique biodiversity in natural systems (medicinal plants). They are an important ecosystem for isolation of LAB **(Siezen *et al.*, 2008; Cakir, 2010; Venugopalan *et al.*, 2010; Baradaran *et al.*, 2012)**. Each specific plant species provides a unique environment in terms of competing microorganisms, natural plant antagonists, as well as accessibility, type and concentration of the substrate in the various physical factors. These conditions allow for the growth of typical epiphytic flora, which gives rise to a population and a chain of fermentation processes when the plant material is collected and prepared for fermentation.
Currently, the available information about the use of medicinal plants as a source of LAB and their subsequent potential application as components to form starters for fermented milks is scarce **(Venugopalan *et al.*, 2010)**. There are no data in the scientific literature about microbial presence of different types of bacteria, in particular LAB, isolated from representatives of the genus *Salvia*. Different types of this medicinal plant are grown and used worldwide as a spice in cooking and in traditional and folk medicine because of their antibacterial, antioxidant, anti-inflammatory and analgesic properties **(Ibrahim *et al.*, 2012)**. A recent new direction in scientific research is related to: obtaining bioactive or biogenic substances extracted from different plants or synthesized during food fermentation; subsequent creation of novel foods (defined as healthy and functional) by additional introduction into their technological schemes of exogenous functional components or use of microorganisms, producers of biogenic substances, as well as microorganisms with probiotic characteristics **(Gobbetti *et al.*, 2010)**.
The aim of this study was to prove that representatives of the genus *Salvia* are alternative ecological niche for isolation and identification of LAB with metabolic activities and characteristics, providing subsequent potential possibility for *in situ* cultivation in milk when producing fermented dairy products.

MATERIAL AND METHODS

Collection of plant samples

The plant material from various representatives of the genus *Salvia* was collected from six regions in Bulgaria and one region in Germany as follows: *S. scabiosifolia* Lam. – Rousse region (area "Bazkite", town of Byala) and from Sofia region (experimental field of the Institute of Biodiversity and Ecosystem Research (IBER) - BAS); *S. ringens* Sibth. & Sm. - Sofia region (IBER - BAS), Shumen plateau (Osmar village), Varna region ("Taushan Tepe", Nevsha village); *S. officinalis* L. - Sofia region (IBER - BAS) and Eastern Rhodopes ("Dayma" region, town of Kroumovgrad and "Luda reka" river, town of Ivaylovgrad); *S. tomentosa* Mill. - Sofia region (IBER - BAS); *S. blepharophylla* Brandegee ex Epling - Botanical garden of the Technical University in Dresden, Germany. The samples were taken aseptically, in sterile tubes, and transported under refrigeration to the laboratory for analyses.

Isolation of lactic acid bacteria

Individual parts (flower, leaf and stem) of each plant species were carefully washed in sterile water, then transferred to test tubes with sterile 10% RSM (Reconstituted Skim Milk) (HiMedia, India) and incubated at temperatures – 30 °C and 37 °C for time period defined from the moment of coagulation of milk for the relevant sample. After visual assessment of milk coagulation, gas formation and non-specific odour, the samples were selected for subsequent transfer to M17 broth (Merck, Germany, Darmstadt) and MRS broth (Merck, Germany, Darmstadt), with added 100 µg/mL of cycloheximide (Sigma-Aldrich, USA, St. Louis) **(Hartnett *et al.*, 2002)** in order to prevent fungal growth and to select LAB. Selected samples were cultured under anaerobic conditions in anaerobic jars with Anaerocult A mini system (Merck, Darmstadt, Germany) for 72 h at the above-mentioned temperatures. Broth cultures subjected to serial decimal dilution with 0.85% (w/v) sterilized NaCl solution were plated by spreading 0.1 mL on MRS agar (Merck, Germany, Darmstadt) and M17 agar (Merck, Germany, Darmstadt) plates. The plates were incubated under anaerobic conditions at 30 °C and 37 °C for 72 – 120 h to obtain LAB colonies. The colonies were selected randomly for purification by streaking again and subculturing on fresh agar plates of the isolation media. The purity of isolated single colonies was evaluated microscopically (Micros Pink MC50, Austria).

The abbreviation of the isolated lactic acid strains from the relevant plant samples includes: initials of the relevant plant species; an index indicating the location of the plant species or an initial of a nearby location to the region in question in Bulgaria, or abbreviated spelling of Germany. (For example SsfIB21: Ss – *Salvia scabiosifolia*, f – flower, IB – IBER - BAS, 21 – number of colony).

Identification of lactic acid bacteria

Phenotypic and biochemical characteristics

The initial study of bacterial isolates involved microscopic characterization of the morphology of cells (Micros Pink MC50, Austria) and colonies (shape, color and size) (CETI, Digi Steddy II, Belgium) and subsequent differentiating Gram staining. Biochemical tests (catalase test, oxidase test, reaction to indole) were carried out of selected Gram (+) isolates to establish their affiliation to the group of LAB.

Isolated presumptive LAB - Gram (+), catalase (-), indole (-), oxidase (-), were stored in M17 broth, containing 15% glycerol (Merck, Germany, Darmstadt) at -80 °C for use in subsequent examinations.

The presumptive LAB were tested for production of CO_2 from glucose, by incubation for 72 h at 37 °C.

The lactic acid isomers produced from glucose in M17 broth (pH 6.6) was determined enzymatically using L(+) and D(-) lactate dehydrogenase kit K-DLATE 12/12 (Megazyme International Ireland Ltd), by incubation for 72 h at 37 °C.

The presumptive LAB that manifested high acid-producing activity were tested for growth in M17 broth (pH 6.6) at different temperatures (4 °C, 15 °C, 30 °C, 37 °C, 45 °C and 55 °C) for 72 h, and for growth in M17 broth at different pH values (3.0, 5.0, 7.0, 8.0 and 9.6) at a temperature of 37 °C for 72 h. The level of salt tolerance of presumptive LAB was determined after growth in M17 broth (pH 6.6) in the presence of various NaCl concentrations (3.0%, 4.5%, 6.5%, 8.5% and 10.0%), at 37 °C for 72 h.

For comparative characterization of the above-mentioned properties of presumptive LAB isolated from the genus *Salvia*, the latter were cultivated in parallel with LAB belonging to the collection of the laboratory and isolated from various dairy products or used in our previous studies - *Lactococcus lactis* ssp *lactis* biovar *diacetylactis* LD5 (*L. diacetylactis* LD5), *Streptococcus thermophilus* ST3 (*S. thermophilus* ST3), *Lactococcus lactis* ssp *lactis* LL3 (*L. lactis* LL3), *Lactococcus lactis* ssp *cremoris* LC1 (*L. cremoris* LC1), *Enterococcus faecium* EF4 (*Ent. faecium* EF4).

The preliminary identification of selected presumptive LAB were investigated using API 20 STREP (BioMerieux, Marcy-L`Etolie, France) galleries. The tests were conducted according to the instruction of the manufacturer and the results were read after incubation of strains at 37 °C for 48 – 72 h.

Genotypic characterization

The isolates were grown anaerobically on M17 agar for 48 h at 37 °C. Colonies were suspended in 1 mL Milli - Q water and centrifuged for 1 min at 12000*g*. Genomic DNA was isolated from a pellet by NucleoSpin® Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), following the instruction of the manufacturer. The concentration of the resultant genomic DNA was measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

Fragments of the 16S ribosomal gene were amplified, using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). Each PCR mixture (50 µL) contained a reaction mix of 25µL HotStarTaq *Plus* Master Mix Kit, 2x (Qiagen GmbH, Germany), 1 µL of each primer (10 µM) and 100 ng of DNA template and autoclaved Milli - Q water. The amplification was performed in Mastercycler® pro (Eppendorf, NY, USA), including initial denaturation at 95 °C for 1 min, followed by 33 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, elongation at 72 °C for 100 s, and a final extension for 3 min at 72 °C. The amplified product was cooled at 4 °C. The PCR products (about 1500 bp) were analyzed by 1 % (w/v) agarose gel electrophoresis in 1xTAE buffer (stock 50xTAE: 242 g/L Tris base, 57.1 mL /L acetic acid, 100 mL 0.5M EDTA, pH 8.5) at 80V. The staining was performed in GelRed (Biotium, USA) fluorescent dye (0.05 µg/ mL). The bands were visualized under ULTima 10SI (Hoefer Inc., USA). The sizes of DNA fragments were estimated using a standard 100 bp DNA ladder (Invitrogen, USA). The PCR products were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions.

The sequencing was performed in Eurofins MWG Operon (Germany). The sequence assembly was performed by using software BioEdit. The sequence similarity was evaluated by searching the homology in NCBI database, using BLAST. The results obtained were used to identify isolates to genus or species level. Phylogenetic tree were constructed, using Mega 4.1 software.

Statistical analyses

Data represent the mean values of three independent experiments. The errors of experimental data from the mean values were expressed as standard deviations using Microsoft Excel 2010 program and illustrated as error bars.

RESULTS AND DISCUSSION

Isolation and obtaining bacterial isolates from *Salvia* species and their preliminary identification

As a result of large-scale screening of 800 microbial isolates (derived from flower, leaf or stem of the 5 types of Salvia - *S. Officinalis* L., *S. ringens* Sibth. & Sm., *S. Blepharophylla* Brandegee ex Epling, *S. scabiosifolia* Lam., *S. tomentosa* Mill.), based on visual assessment for milk coagulation, gas formation and non-specific odour, 124 microbial isolates were selected, which is 15.5%. After subsequent multiple transfer and growth in selective media, 460 single bacterial colonies were isolated. They were identified by classical techniques to prove their phenotypic characteristic and their respective affiliation to the group of LAB. The data from the required and confirmatory tests established that 12.2% (56 single colonies) showed phenotypic identity with the group of LAB. They were Gram-positive, catalase-negative, oxidase-negative and indole-negative. The flowers of the plant turned out to be the most favorite part for habitation of LAB (27 single colonies), followed by the leaves (15 colonies) and the stem (9 colonies). Lactobacteria were not isolated only from the species *S. tomentosa* Mill., and the highest number (25 colonies) were isolated from *S. ringens* Sibth. & Sm. LAB were not isolated from *S. officinalis* L. from the areas in the Eastern Rhodopes.

The presumptive LAB were morphologically defined only as cocci (arranged as a single cell, in pairs, in short or long chains) with a cell size from 0.3 µm to 2.0 µm. The relevant colonies are shiny, with a wide variety of colors (white, light beige and yellow), shape (circular - convex or flat, with entire or undulate margins) and size (from 1.1 mm to 3.1 mm).

Each plant species provides unique growth conditions for different types of lactobacteria. **Michaylova *et al.* (2007)** have discovered that plant species *Calendula officinalis, Capsella bursapastoris, Chrysanthemum, Cichorium intybus, Colchicum, Cornus mas, Galantus nivalis, Dianthus, Hedera, Nerium oleander, Plantago lanceolata, Prunus spinosa, Rosa and Tropaeolum* are suitable sources for isolating cocci, and the species *Calendula officinalis, Cornus mas, Galantus nivalis* and *Prunus spinosa* for isolation of both cocci and rods. From naturally fermented herbs used in traditional Herby Cheese in Turkey predominantly lactobacilli (76.2%) vs 23.8% cocci were isolated **(Cakir, 2010)**, and only rods were isolated from the herbal surface of *Phyllanthus niruri* **(Venugopalan *et al.*, 2010)**. Cocci and rods were isolated from the surface of *Polygonum minus* leaves, a Malaysian local herb (in a ratio 2:1) **(Baradaran *et al.*, 2012)** and from different plants (Clover, Grass, Dandelion, Lilac flowers, Chestnut flowers, Hapatica flowers, Coltsfoot flowers and Rowan leaves) **(Magnusson *et al.*, 2003)**. Cocci were isolated from twenty grass varieties and vegetables **(Alemayehu *et al.*, 2014)**.

Regarding the ability of the isolated lactobacteria to produce CO_2 from glucose, all tested isolates showed absence of such metabolic activity and hence they were referred to the group of homofermentative LAB.

Determination of isomeric forms of produced lactic acid, degree of halotolerance, and temperature and pH ranges for growth of bacteria

A significant characteristic of LAB intended for the dairy industry is their acid-producing activity, i.e. production of lactic acid (LA) during their homofermentative or heterofermentative metabolism. In this regard, LA production was studied and isomeric forms were determined during the homofermentative metabolism of presumptive LAB isolated from *Salvia*. The data for isomeric forms of LA produced by plant lactobacteria showed that 88.2% synthesize L(+)-LA, and 11.8% D(-)/L(+)-LA (Fig 1A, B). Moreover, in the isolates producing both isomeric forms of LA the presence of L(+)-LA is dominant. There were no representatives producing only D(-)-LA. The highest activity of LA synthesis was observed in the isolates obtained from *S.*

blepharophylla Brandegee ex Epling, with attained maximum concentration of 10.01 g/L and 15.56 g/L (Fig 1A). One half of the *S. scabiosifolia* Lam. isolates also showed a high acid-producing activity, with LA concentration in the range of 7.74 g/L - 10.29 g/L (Fig 1A). Relatively lower concentrations of LA (about 4.00 g/L - 5.00 g/L) were found in *S. ringens* Sibth. & Sm. isolates (Fig 1B).

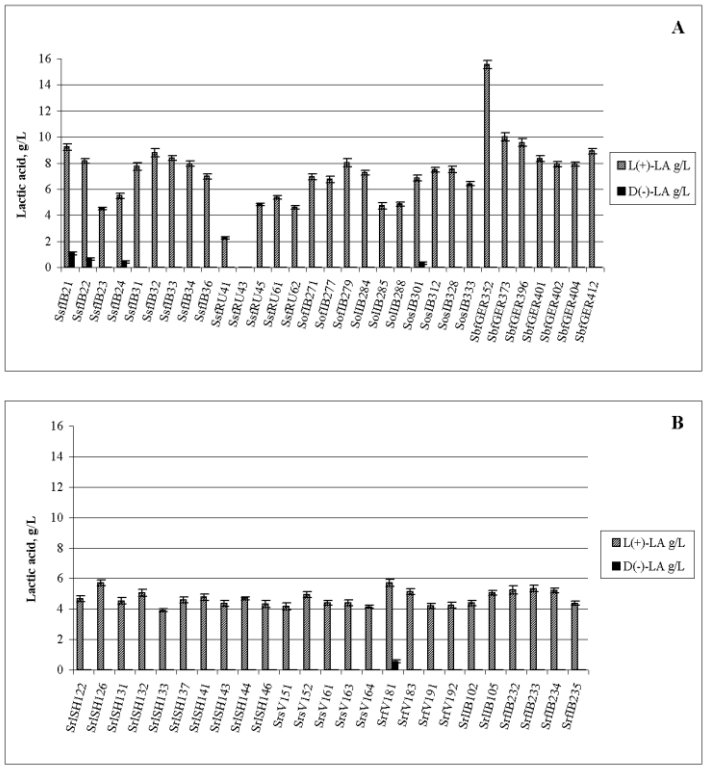

Figure 1 Isomeric forms of lactic acid produced by lactic acid bacteria, isolated from *Salvia* species: A – *S.scabiosifolia* Lam. (Ss), *S. officinalis* L. (So), *S. blepharophylla* Brandegee ex Epling (Sb); B – *S.ringens* Sibth. & Sm. (Sr): ▨ - L(+)-lactic acid; ■ - D(-)-lactic acid

The observed concentrations of LA produced by selected LAB of plant origin are comparable to those produced by lactobacteria isolated from dairy products – *L. diacetylactis* LD5 - 10.00 g L(+)-LA/L, *L. lactis* LL3 - 8.08 g L(+)-LA/L, *L. cremoris* LC1 - 7.47 g L(+)-LA/L, *S. thermophilus* ST3 - 9.39 g L(+)-LA/L, *Ent. faecium* EF4 - 6.85 g L(+)-LA/L (Fig 2).

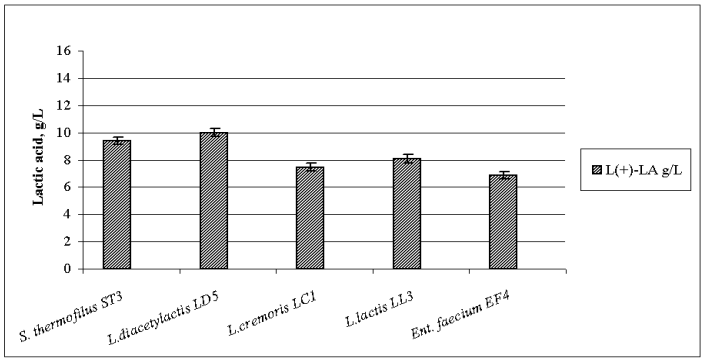

Figure 2 Isomeric forms of lactic acid produced by lactic acid bacteria, isolated from dairy products: ▨ - L(+)-lactic acid

Similar results were reported by **Kimoto *et al.* (2004)**, who obtained 20 raw grass bacterial isolates, 'napiergrass' in Japanese, thereafter morphologically defined and tested as homofermentative cocci, producing only L(+)-LA. **Kostinek *et al.* (2007)** have also isolated homofermentative cocci from fermented cassava, producing only L(+)-LA. Other researchers have isolated homofermentative cocci, producing only D(-)/L(+)-LA, from traditionally fermented vegetable products of the Eastern Himalayas **(Tamang *et al.*, 2005)** and from fermented tea leaves (miang) in Thailand **(Tanasupawat *et al.*, 2007),** respectively. Unlike our data, heterofermentative cocci producing only D (-)-LA have also been isolated from the above plant sources with the exception of fermented tea. **(Tamang *et al.*, 2005; Kostinek *et al.*, 2007)**. Besides determination of isomeric forms of LA synthesized by presumptive lactobacteria, some authors have also reported data on LA concentrations as an element of their pre-identification. In this regard, the results reported by **Cock and De Stouvenel (2006)** are of interest, who, out of 20 selected bacterial isolates obtained from sugar molasses leaves, selected only one strain (with index CC 85-92), with a high potential for synthesis of L(+)-LA (12.4 g/L and 13.7 g/L, at cultivation temperatures 36 °C and 32 °C, respectively). These data are comparable with the results obtained for the isolates in this study (Fig 1A, B). With this strain, cultivated in parameters suitable for targeted synthesis of LA, the authors reached the maximum yield of 35.0 g/L.

Growth parameters (temperature, pH and presence of NaCl) of LAB are essential physiological parameters for the phenotypic characteristic of the relevant type, showing aspects for their subsequent application in various technological schemes. Almost all isolates showed very good growth in a wide temperature range (15 °C – 45 °C), good and weak growth at 4 °C and absence of growth at 55 °C (Table 1). The pH range for growth of most isolates was also extended, i.e. very good growth was obtained in the range of 7.0 - 9.6, good growth at pH 5.0, and absence of growth at pH value of 3.0 (Table 1). Data for a wide temperature range of growth (15 °C - 45 °C) and (10 °C – 45 °C) were reported by **Tanasupawat *et al.* (2007)** and **Baradaran *et al.* (2012)** for lactococci isolated from fermented tea leaves and from the herb *Polygonum minus*, respectively. The same authors established that the isolates studied by them grow well also within a pH range of 3.0 - 7.0 and 4.0 - 8.5, which is in accordance with the results reported by **Tamang *et al.* (2005)** for lactococci of other plant origin (fermented vegetable products). **Alemayehu *et al.* (2014)** proved good growth at pH 9.5 of lactococci isolated from grass varieties and vegetables. The studied plant isolates showed better growth characteristics in a broader temperature and pH range in comparison to those of LAB of dairy origin (Table 1). A similar trend was recorded for their halotolerance (Table 1). The major part of plant isolates showed very good growth in a medium containing NaCl, at concentrations from 3.0% to 6.5%. At 8.5% concentraction of NaCl, about 1/2 of the isolates showed good growth. 10% NaCl concentration proved to be inhibitory for nearly all isolates. **Tamang *et al.* (2005), Baradaran *et al.* (2012)** and **Alemayehu *et al.* (2014)** established a high level of halotolerance (growth in the presence of 10% NaCl and 6.5% NaCl), but **Kimoto *et al.* (2004)** and **Tanasupawat *et al.* (2007)** found a lower level (growth in the presence of 4.0% NaCl) for lactococci of different plant origin.

Identification of lactic acid bacteria by PCR and constructing of a phylogenetic tree

Phenotyping of LAB using morphological, physiological and biochemical (API 20 STREP) characteristics identified representatives of several species belonging to the genera *Lactococcus* and *Enterococcus* with a similarity of about 60% and 80%, respectively. The identification system used did not give 100% accuracy of identity.

Genotyping is known to give a surer identity of microorganisms. Basically genotype-based methods such as 16S rDNA sequencing represent a successful addition to phenotypic methods for precise identification of lactobacteria isolated from certain microbial communities **(Kostinek *et al.*, 2007; Tanasupawat *et al.*, 2007; Chen *et al.*, 2010; Kpikpi *et al.*, 2010; Baradaran *et al.*, 2012; Venturi *et al.*, 2012; Alemayehu *et al.*, 2014**). Although LAB are widespread, each plant habitat is characterized by a specific microbocenosis, having a different biological activity. The isolated DNA from phenotypically characterized bacterial isolates was used as a matrix for PCR amplification of 16S rDNA genes. The combination of PCR amplification of 16S rDNA genes, sequencing of the resulting PCR products and the subsequent comparison of their sequences with the ones existing in the database NCBI allowed a precise identification of the presumed LAB to a species level, as well as their phylogenetic grouping. The majority (69.0%) of isolates analyzed showed affiliation to the genus *Enterococcus*. Full compliance of phenotypic characteristic and the results of PCR analysis with a nearly 100% homology of the nucleotide sequence allowed us to relate the strains SofIB271, SofIB279, SofIB284, SofIB328 to the species *Ent. faecium*. The strains SsfIB21, SsfIB22, SsfIB32, SsfIB33, SsfIB34, SofIB277, SosIB301, SosIB312, SrsV161 are also related to the same species and demonstrated a high level of homology - 99%. The strains SrlSH132, SrlSH141, SrlSH144 demonstrated the same homology (99%) as both species – *Ent. casseliflavus* and *Ent. gallinarum*, but the phenotypic difference between the two species regarding the color of the colony, yellow and white, respectively, determined the affiliation of the isolates to *Ent. casseliflavus*. Strains SsfIB24, SrsV151, SolIB288 were identified as *Ent. mundtii* with 99% homology to the nucleotide sequence and SolIB285 - 100%.

A minor part, (20.7% and 10.3%) of the isolates analyzed were identified as representatives of the genus *Lactococcus* and the genus *Streptococcus*, respectively. A high level of similarity (99.0%) with *L. lactis* subsp *lactis* was recorded for the strains SsfRU61, SsfRU62, SrfV183, SrfIB232, SrfIB233, and for the strain SrfV181 - 100% similarity. A 99% homology of the nucleotide sequence allowed us to relate strains SbfGER352, SbfGER373, SbfGER401 to the species *S. thermophilus.*

Evidence of genotyped representatives of the species of the genera *Enterococcus, Lactococcus, Streptococcus* established in this study in isolates of different origin have also been reported by other authors **(Hartnett *et al.*, 2002; Kimoto *et***

***al.*, 2004; Cock and De Stouvenel, 2006; Nomura *et al.*, 2006; Michaylova *et al.*, 2007; Baradaran *et al.*, 2012)**. **Hartnett *et al.* (2002)** reported about identified *Ent. faecium* and *L. lactis* in isolates from raw barley, and in isolates from sorghum were defined the species *Ent. mundtii* and the species *Ent. faecalis* – undetected in studied by us plant isolates from *Salvia*. The species *L. lactis* was isolated and identified from a type of Japanese raw grass (napiergrass) **(Kimoto *et al.*, 2004; Nomura *et al.*, 2006)** and from the leaves of sugar beet **(Cock and De Stouvenel, 2006)**. Except *L. lactis*, **Baradaran *et al.* (2012)** *Pediococcus pentosaceus* and *Lactobacillus curvatus* were defined in isolates of *Polygonum minus* (a Malaysian herb). *S. thermophilus* has been isolated from the leaves of 14 different plant species, and in isolates from 4 plant species *Lactobacillus bulgaricus* has also been identified **(Michaylova *et al.*, 2007)**. *L. lactis* ssp *lactis* and *L. lactis* ssp *cremoris* **(Alemayehu *et al.*, 2014)** were isolated from grass varieties and vegetables. The summarized data reveal that each of the studied plant habitat represents a separate ecological niche for growth of specific lactic acid microflora.

Based on data from conducted 16S rDNA gene sequence analysis, using computer program Mega 4.1, it was constructed a phylogenetic tree, which displays a high consistency regarding relationships between the studied strains (Fig 3). The phylogenetic tree shows the diversity of the isolated plant – derived lactic acid bacteria, belonging to genera *Enterococcus*, *Streptococcus* and *Lactococcus*, as well as it displays the phylogenetic position of the isolates, divided into several clusters and sub-clusters. It also demonstrates nice patterns of the isolates based on geographical regions and plant parts, where they were isolated from.

Table 1 Effect of temperature, pH and NaCl concentration on survival of bacterial isolates and LAB of dairy origin

Isolates	Temperature (°C)						pH					NaCl (%)				
	4	15	30	37	45	55	3.0	5.0	7.0	8.0	9.6	3.0	4.5	6.5	8.5	10
SsfIB21	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	-
SsfIB22	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	+^{w}
SsfIB24	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	-
SsfIB32	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	-
SsfIB33	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+^{w}	+^{w}
SsfIB34	+	++	++	++	++	-	-	+	++	++	+	++	++	++	+	-
SsfRU61	+	++	++	++	-	-	-	+	++	++	+	++	+	-	-	-
SsfRU62	+	++	++	++	-	-	-	+	++	+	+	++	+	-	-	-
SrlSH132	+^{w}	+	++	++	++	-	-	+	++	++	++	+	+	+	+ -	-
SrlSH141	+	++	++	++	++	-	-	+	++	++	++	++	++	+	-	-
SrlSH144	+	++	++	++	++	-	-	+	++	++	++	+	+	+^{w}	+^{w}	-
SrsV151	+	++	++	++	++	-	-	++	++	++	++	++	++	++	+	+
SrsV161	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	+
SrfV181	+^{w}	+	++	++	+^{w}	-	-	+	++	++	+	+	+	-	-	-
SrfV183	+	++	++	++	+^{w}	-	-	+	++	++	++	++	++	+	-	-
SrfIB232	+	+	++	++	+^{w}	-	-	+	++	++	+	++	++	+	-	-
SrfIB233	+^{w}	+	++	++	+^{w}	-	-	+	++	+	+	++	+	+	+	-
SofIB271	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	-
SofIB277	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	-
SofIB279	+	++	++	++	++	-	-	+	++	++	++	++	++	++	++	+^{w}
SolIB284	+	++	++	++	++	-	-	+	++	++	+	++	++	++	+	-
SolIB285	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	-
SolIB288	+	++	++	++	++	-	-	+	++	++	+	++	++	++	+	-
SosIB301	+	++	++	++	++	-	-	+	++	++	+	++	++	++	+	+^{w}
SosIB312	+	++	++	++	++	-	-	+	++	++	++	++	++	++	+	+^{w}
SosIB328	+	++	++	++	++	-	-	+	++	++	++	++	++	++	++	-
SbfGER352	+^{w}	+^{w}	++	++	++	-	-	+	++	++	+	-	-	-	-	-
SbfGER373	+^{w}	+^{w}	++	++	++	-	-	+	++	++	+	-	-	-	-	-
SbfGER401	+^{w}	+^{w}	++	++	++	-	-	+	++	+	+	-	-	-	-	-
***L.lactis* LL3**	+^{w}	+^{w}	++	++	-	-	-	+	++	+	+^{w}	++	+	+^{w}	-	-
***L.cremoris* LC1**	-	+^{w}	++	++	-	-	-	+	++	+	-	+	-	-	-	-
***L.diacetylactis* LD5**	+^{w}	+	++	++	-	-	-	+	++	+	+^{w}	+	+	+^{w}	-	-
***S.thermophilus* ST3**	+^{w}	+^{w}	++	++	++	+^{w}	-	+	++	+	+^{w}	+^{w}	+^{w}	+^{w}	-	-
***E.faecium* EF4**	+	++	++	++	++	+^{w}	-	+	++	++	++	++	++	+	+	+^{w}

Growth: ++ very good; + good; - no; ^{w} weak;

Figure 3 Phylogenetic tree based on the 16S rDNA gene sequences, of identified isolates from medicinal plants (representatives of genus *Salvia*), determined using the neighbor-joining method

CONCLUSION

The results of this study show that representatives of the genus *Salvia (S. officinalis, S. ringens* Sibth. & Sm., *S. Blepharophylla* Brandegee ex Epling, *S. scabiosifolia* Lam.) are appropriate ecological niches for isolation of various types of lactococci, unlike the species *S. tomentosa* Mill. The better growth characteristics of the isolated and identified lactococci (wide pH and temperature range) as well as their higher acid-producing activity, comparable to LAB of dairy origin, are an advantage and a good prerequisite for *in situ* cultivation in milk and subsequent formation of starter cultures for new fermented diary products.

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